A DEOXYRIBONUCLEASE FROM CALF SPLEEN*

II. MODE OF ACTION

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When highly polymerized deoxyribonucleic acid[1] is digested with pancreatic DNase and the digest further degraded with phosphodiesterase from intestinal mucose or snake venom, the mononucleotides formed are 5′-phosphates (1, 2). Since a digest prepared in this manner with a purified venom diesterase contains only 5′-mononucleotides and practically no nucleosides or polynucleotides (3, 4), the pancreatic DNase must form only polynucleotides with 5′-monoesterified phosphate end groups. Recently the enzymatic synthesis of polydeoxyribonucleotides from deoxyribonucleoside 5′-triphosphates has been demonstrated (5). Thus both enzymatic synthesis and degradation of deoxyribonucleic acids have as yet involved only derivatives with 5′-monoesterified phosphate.

This paper presents evidence that the DNase isolated from spleen (6) yields polynucleotides with 3′-monoesterified phosphate end groups. Recent data by Cunningham, Catlin, and Privat de Garilhe (7) suggest that the DNase from *Micrococcus pyogenes* may act in a similar manner.

Materials and Methods

Splenic DNase was prepared by the method described in Paper I of this series (6).

DNA used in this study was from a single preparation of calf thymus DNA. The preparative procedure was essentially the "Method A" of Marko and Butler (8), scaled up for 1200 gm. of thymus tissue, and with

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[1] The following abbreviations are used: DNase, deoxyribonuclease; DNA, deoxyribonucleic acid; T-A buffer, a buffer composed of tris(hydroxymethyl)aminomethane and acetate; the molarity refers to the total concentration of tris(hydroxymethyl)aminomethane.

the centrifugations performed in a Sharples centrifuge. The resulting DNA was of lower viscosity than some preparations made by the method of Mirsky and Pollister (9), and also yielded a small white precipitate when degraded with either pancreatic or splenic DNase. (This precipitate presumably represents a small amount of unextracted protein.) Pancreatic DNase digests from this preparation and from preparations made by the method of Mirsky and Pollister were identical both as to the end point of digestion and to products isolated by ion exchange chromatography (10).

Prostatic phosphatase was generously supplied by Dr. Gerhard Schmidt. This enzyme can be assayed by the same procedure as that used for splenic acid phosphatase (6).

The method of preparation of venom phosphodiesterase previously described (4) does not provide solutions of sufficient specific activity for convenient use against the highly resistant substrates encountered in this study. A new acetone precipitation procedure was therefore devised which yields preparations of adequate concentration and freedom from 5′-nucleotidase. These preparations were assayed by a more convenient and rapid modification of the earlier assay method

Assay for Venom Phosphodiesterase—A substrate solution of 10 ml. of calcium bis(*p*-nitrophenyl)phosphate, 3 ml. of 0.3 M magnesium acetate, 10 ml. of 0.1 M T-A buffer, pH 8.8, and 6 ml. of water was made within a few hours of use. The calcium bis(*p*-nitrophenyl)phosphate solution was 0.001 M in the reagent salt and 0.01 M in ammonium acetate buffer, pH 4.5. (This acidic solution will keep for many weeks in the cold, whereas, if basic, the substrate deteriorates rapidly.)

2.9 ml. of the substrate solution were added to a spectrophotometer cuvette and warmed to 37° in a cuvette housing attachment with thermostat. The sample to be assayed (not greater than 0.1 ml.) was stirred in and the absorbance at 440 mμ read. After 10 minutes incubation, the A_{440} was read again and the difference between the two readings, multiplied by 1000, was recorded as the total phosphodiesterase activity of the sample. This assay was linear with respect to concentration to an activity of about 500 units.

Purification of Venom Phosphodiesterase—A water solution of 16.7 mg. of lyophilized *Crotalus adamanteus* venom per ml. of water was left at room temperature for an hour and then centrifuged. (This and subsequent centrifugations were carried on for 15 minutes at 4000 to 5000 r.p.m.) To 1 volume of the clear supernatant solution was added 0.67 volume of 0.50 M ammonium acetate buffer (0.50 M in total acetate), pH 4.00, and this buffered venom solution was stored in the cold room (2°), where all subsequent procedures were carried out.

10.0 ml. of buffered venom were added to a centrifuge tube, and 7.25 ml. of reagent grade acetone at −20° pipetted rapidly on to this solution. The tube was covered tightly with aluminum foil and swirled rapidly to mix the reagents. After it stood for 30 minutes, the mixture was centrifuged and the supernatant liquid was decanted into another centrifuge tube. (A slightly turbid supernatant liquid at this stage was occasionally observed.) 1.4 ml. of acetone were added and the solution was stored for 12 hours with the tube stoppered. It was then centrifuged and the clear supernatant solution decanted into another centrifuge tube. 1.2 ml. of acetone were added and this solution was centrifuged after being allowed to stand for 30 minutes. The final active precipitate was dissolved in water.

TABLE I

Purification of Venom Phosphodiesterase

Solution	Volume of sample	Activity	Biuret assay (11) on 0.025 ml. sample (A_{544})
	ml.	*units*	
Buffered venom solution	0.025	215	0.415
Supernatant from 1st acetone precipitation	0.050	240	
" " 2nd " "	0.050	200	
" " 3rd " "	0.050	26	
Water solution of purified phosphodiesterase	0.010	384	0.220

This method was capable of excellent results, but occasionally a preparation would contain a large amount of 5′-nucleotidase. For this reason, several tubes were prepared simultaneously and assayed individually for 5′-nucleotidase. 50 μl. of a satisfactory final solution would release less than 1 γ of inorganic phosphate from adenosine 5′-phosphate when incubated for 12 hours under the conditions previously described (4). Table I summarizes the results of this preparation.

Determination of Monoesterified Phosphate in DNase Digests—The number of phosphate diester bonds hydrolyzed by pancreatic DNase can be easily determined by addition of standard base to neutralize the acid released by the formation of secondary phosphate (12–16). However, since the splenic DNase degradation must be run in acidic solution, the secondary phosphate released is not ionized and does not affect the pH of the solution. In addition, many amino groups on the purine and pyrimidine bases become ionized during the degradation, and liberate hydroxyl ions. The secondary phosphate released can be crudely determined by adding standard acid to maintain the pH at 4.5 during the digestion, titrating through the pH region of amino and secondary phosphate ionization with

standard base, and subtracting the moles of hydroxyl ion released by the amino groups from the total moles titrated. In addition, a small correction for the moles of base needed to titrate the undegraded DNA is necessary. Although such a determination has many uncertainties, the results obtained have agreed reasonably well with the results obtained more directly by an enzymatic determination of monoesterified phosphate.

The acid-prostatic phosphatase of Schmidt (17) is free from diesterase activity against polynucleotides from pancreatic DNase digests, and has been used to remove the monoesterified phosphate from the small polynucleotides of such digests during structural determinations (18). However, larger polynucleotides form complexes with the inorganic phosphate after its release, which interfere with the determination of inorganic phosphate by the usual phosphomolybdate procedure.[2] Therefore a modified inorganic phosphate determination, patterned after the method of Lowry and Lopez (19) but carried out at an elevated temperature to break the polynucleotide-inorganic phosphate complex, has been employed. By release of the monoesterified phosphate with prostatic phosphatase and determination of the inorganic phosphate in this manner, it is possible to obtain a measure of the monoesterified phosphate arising during a DNase digestion.[3]

During degradation by splenic DNase, the results obtained by this method agreed with results obtained by the cruder titration method which has been described. As will be seen, these results also agree with a third estimate of end groups, obtained without use of phosphatase, by means of degradation of the polynucleotide mixture with a concentrated venom phosphodiesterase preparation.

A measured volume of DNase digest, estimated to contain 5 to 10 γ of monoesterified phosphate, 0.050 ml. of prostatic phosphatase, 1 ml. of 0.2 M ammonium acetate buffer, pH 5.5, and water to make a solution volume of 1.5 ml. was incubated for 4 hours[4] at 37°. 1 ml. of 0.5 M ammonium acetate buffer, pH 4.0, 0.3 ml. of 1 per cent ascorbic acid, and 0.3 ml. of 1 per cent ammonium molybdate in 0.05 N sulfuric acid were added. The solution was placed in a 60° water bath for 20 minutes and then allowed to stand at room temperature for 30 minutes, after which the absorbance at 825 mμ was read.

[2] These polynucleotide-phosphate complexes are sufficiently stable that the inorganic phosphate will not appear in its customary place on an anion exchange resin chromatogram of such a mixture.

[3] Control experiments with digests produced by pancreatic DNase yielded results by this method which were in good agreement with the data obtained by titration of the acid released during the degradation.

[4] This monoesterified phosphate procedure yields constant results with prostatic phosphatase incubation times ranging from 45 minutes to 16 hours.

A phosphatase control (the same as above, except that water replaced the DNase digest), DNase digest control (as above, except that water replaced the phosphatase), standards (as above, except that known amounts of inorganic phosphate replaced the phosphatase), and a blank (water replaced both the phosphatase and the DNase digest) were made up at the same time as the digest, and all determinations were carried through the entire procedure simultaneously. Appropriate controls and blank were subtracted from all determinations. Total phosphate was determined in the DNase digest by hydrolysis to inorganic phosphate by the perchloric acid procedure of Allen (20), followed by a phosphomolybdate determination of the inorganic phosphate by a procedure similar to that of Ammon and Hinsberg (21), by use of ascorbic acid (1 per cent) as a reducing agent for 20 minutes at 60°.

Kinetics of Degradation of DNA—All DNase digests were carried out with a concentration of 10 mg. of DNA per ml. and in a solution 0.02 M in ammonium acetate buffer, pH 4.5, and 0.15 M in sodium chloride. The DNA and the stock buffer and sodium chloride solutions were dissolved in sufficient water that the final concentrations would be at these values after addition of the DNase solution. At this concentration of DNA, the resulting solution was a heavy gel. DNase solution of the desired amount was added, and the mixture was incubated at 37° with frequent shaking until the substrate had liquefied. The small precipitate derived from the contaminant in the DNA was then removed by centrifugation, and the incubation was continued. At various times, appropriate aliquots of the digest were removed and added to an amount of tris(hydroxymethyl)-aminomethane solution calculated to raise the pH to approximate neutrality. The mouths of the tubes containing these samples were sealed with aluminum foil and the tubes were heated on an 80° water bath for 20 minutes to inactivate the DNase.[5] Then the monoesterified phosphate was determined as previously described.

Results

Kinetic Studies—Fig. 1 presents the results of experiments with different concentrations of the purified DNase. There is evidently a rapid degradation of DNA until about 10 per cent of the phosphate links is hydrolyzed. Further degradation at a rate which is more than an order of magnitude

[5] If the thermal inactivation of the DNase is carried out at pH 4.5, a small, but detectable amount of purine bases is hydrolyzed from the polynucleotides. Heating in neutral solution, under the conditions given, has no detectable effect on the polynucleotides. This has been verified by comparative analyses of heated and unheated aliquots of a solution in which the DNase digestion had been continued for many hours, so that further DNase attack during the analysis of the unheated control was negligible.

less than the initial rate of hydrolysis occurs until approximately 20 per cent of the links is hydrolyzed. At very high enzyme concentration still further degradation occurs, and, although in one experiment over 30 per cent of the phosphate links was hydrolyzed, no definite end point has been observed for the hydrolysis.

The continued slow release of monoesterified phosphate could be an action of the splenic DNase or could be caused by a contaminating phosphodiesterase which continued to release end groups slowly after the DNase action was complete. In order to decide between these possibilities, the

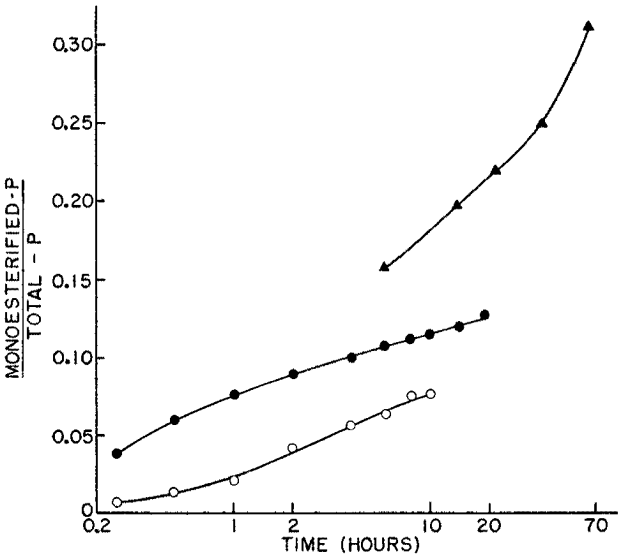

FIG. 1. Degradation of DNA by splenic DNase. ○, 500 units of DNase per ml.; ●, 5000 units of DNase per ml.; and △, 100,000 units of DNase per ml.

rate of slow release of monoesterified phosphate (between a monoesterified phosphate to total phosphate ratio of 0.1 and 0.2, as illustrated in Fig. 1) was compared with the rate of the initial DNase action, as determined by the rate of increase of ultraviolet absorption of the DNA substrate, for various DNase preparations. If the slow degradation is produced by the DNase, its rate would be the same at a given DNase concentration, as measured by the method of increase of ultraviolet absorption, in DNase samples from different stages of purification and with differing DNase to phosphodiesterase activity ratios. This has been found to be the case in a number of preparations. A solution of DNase after the ethanol precipitation step, with a DNase to phosphodiesterase activity ratio of 10,000, gave the same rate of slow degradation as a sample after Celite chroma-

tography with a DNase to phosphodiesterase ratio of 180,000 (at equal levels of DNase activity).

That the phosphodiesterase activity which remains with the DNase after the Celite chromatography is not responsible for the slow degradation can be demonstrated by comparison of preparations in which the DNase has been selectively partially inactivated after the chromatography step. (Such preparations were accidentally obtained at times by use of concentration procedures in the absence of ethylenediaminetetraacetate.) At a given DNase concentration, the same rates of slow degradation were obtained with such preparations for which the DNase to phosphodiesterase ratio varied from 40,000 to 180,000. Since the slow degradation is independent of the phosphodiesterase concentration assayed by the synthetic substrate, and since it parallels DNase activity during purification procedures and partial inactivation of the DNase, it is considered to be a property of the DNase and not of a contaminant.

The very slow final stages of the degradation shown in Fig. 1 are obtained at such a great DNase concentration that small amounts of contaminants in the preparation could have detectable effects. Since residual phosphatase activity is detectable at these concentrations, the possible role of phosphodiesterase activity was not examined.

End Group Studies—To obtain information about the position of the bonds hydrolyzed by the DNase and the specificities of the enzyme, if any, the DNase action was halted at various degrees of degradation, and the terminal groups present in the resultant polynucleotide mixtures were determined by means of further degradation with prostatic phosphatase and with venom phosphodiesterase, with chromatographic analyses of the final digests.

Table II summarizes the results of five such experiments. DNA solutions with a DNA concentration of 10 mg. per ml., 0.02 M in ammonium acetate buffer, pH 4.5, 0.15 M in sodium chloride, and with the desired concentration of splenic DNase were incubated to attain the desired ratio of monoesterified phosphate to total phosphate, by consideration of the results of previous experiments. After incubation, the digests were adjusted to neutrality with tris(hydroxymethyl)aminomethane and heated on an 80° water bath for 20 minutes to inactivate the DNase. If phosphatase digestions were to be carried out, the pH was then adjusted to 5.5, 0.050 ml. of phosphatase was added per ml. of DNase digest, and the solution was incubated for 4 hours at 37°. The phosphatase was then inactivated by shaking for 30 minutes with ether, with subsequent extraction of the ether with spectroscopic grade cyclohexane. Phosphodiesterase digestions were carried out at pH 9.2 at 37° in the presence of 0.01 M magnesium ion. If a large amount of phosphodiesterase was used, the

TABLE II

Enzymatic Degradations of Splenic DNase Digests

The conditions of phosphodiesterase digestion were, for Experiments 1 and 2, 100 units per ml., 26 hours, 37°; for Experiment 3, 4000 units per ml., 26 hours, 37°; for Experiment 4, none; and for Experiment 5, 4000 units per ml., 48 hours, 37°.

	Experiment 1 (DNase, phosphodiesterase)	Experiment 2 (DNase, phosphatase, phosphodiesterase)	Experiment 3 (DNase, phosphodiesterase	Experiment 4 (DNase)	Experiment 5 (DNase, phosphodiesterase)
	Monoesterified P divided by total P after DNase action				
	0.077	0.077	0.10	0.32	0.32
	Substances isolated*				
Nucleosides†	C = 1.9 T = 3.6 A = 1.3 G = 2.3	C = 2.3 T = 2.2 A = 2.0 G = 3.2			
Total.....	9.0	9.8	10.0	1.5	14.0
Nucleotides†	C5′ = 14.5 T5′ = 14.7 A5′ = 12.6 G5′ = 7.8	C5′ = 17.9 T5′ = 25.0 A5′ = 25.5 G5′ = 16.5	C5′ = 14.8 T5′ = 22.2 A5′ = 19.6 G5′ = 12.0	C3′ + T3′ = 4.2 A3′ + G3′ = 6.7	C5′ = 5.4 C3′ = 3.2 T5′ = 10.9 T3′ = 1.3 A5′ + A3′ = 8.2 G5′ + G3′ = 6.3
Total.....	50	85	69	10.9	36

Nucleoside diphosphates†			C di-P = 0.5 T di-P = 1.9 A di-P = 2.0 G di-P = 2.2		C di-P + T di-P = 2.1 A di-P = 5.1 G di-P = 2.3
Total			6.6		9.5
Total polynucleotides			4.1		
Total moles‡	59	95	89		59

* C = deoxycytidine; C5′ deoxycytidine 5′-phosphate; C3′ deoxycytidine 3′-phosphate; C di-P deoxycytidine 3′,5′-diphosphate. T = thymidine; T5′ thymidine 5′-phosphate; T3′ thymidine 3′-phosphate; T di-P thymidine 3′,5′-diphosphate. A = deoxyadenosine; A5′ deoxyadenosine 5′-phosphate; A3′ deoxyadenosine 3′-phosphate; A di-P deoxyadenosine 3′,5′-diphosphate. G = deoxyguanosine; G5′ deoxyguanosine 5′-phosphate; G3′ deoxyguanosine 3′-phosphate; G di-P deoxyguanosine 3′,5′-diphosphate.

† Moles per 100 moles of P.

‡ Identified per 100 moles of P.

digest, after incubation, was heated to 80° for 20 minutes and then centrifuged to remove the bulk of the protein which would otherwise interfere with the subsequent determinations. The digests were fractionated on Dowex 1-×8 anion exchange resin columns by the methods[6] previously described (23, 24).

The molar fraction of nucleotide residue in each column fraction was calculated from the ratio of the moles of monomer in each fraction to the moles of total phosphate in the digest. The moles of known compounds were determined from their published molar absorbancies (10). The molarities of unresolved mixtures and unknown polynucleotides were calculated by assuming a molar absorbancy at 270 mμ of 10,500. Since the absorbancies of all of the major mononucleotides are within ±5 per cent of this value, this average should give results within other experimental errors.

DISCUSSION

Possible Modes of DNase Action—If we assume that splenic DNase catalyzes the hydrolysis of DNA without any rearrangement of nucleotide sequence or structure and (as observed) without release of inorganic phosphate, we might conceivably find, among the products of digestion, polynucleotides with either 3′- or 5′-terminal phosphates, with both 3′- and 5′-terminal phosphates, or with no terminal phosphate. The presence and relative proportions of such products can be deduced from analyses of the products of further degradations with phosphatase, with phosphatase and then venom phosphodiesterase, and with venom phosphodiesterase alone. The conclusions to be drawn from such analyses will rest upon the assumption that venom phosphodiesterase will break all phosphodiester bonds in the polynucleotides at the links to the 3′ positions. That this is the case for polydeoxyribonucleotides with a 5′-terminal phosphate and for those with no monoesterified phosphate has been verified by studies on the polynucleotides from pancreatic DNase digests (18). That it will do so with polydeoxyribonucleotides with a 3′-terminal phosphate and with those with both a 3′ and 5′ end group is less certain. Heppel, Ortiz, and Ochoa (25) and Cohn and Volkin (26) report that small polyribonucleotides with 3′-terminal phosphate are resistant to this enzyme.

When the DNase digestion is carried to the extent used for Experiments

[6] To separate the nucleosides, the nucleoside fractions from the column were lyophilized, dissolved in water, and separated by descending paper chromatography by use of a butanol-ammonia-water system (22). The nucleosides were located by viewing the dried paper under an ultraviolet light. The individual spots were cut out, eluted with 5 ml. of 0.05 M ammonium acetate buffer at pH 4.3 and their absorption spectra were measured by using as a blank samples of the paper of equal area and eluted under the same conditions.

1, 2, and 3, no significant absorption is eluted in an ion exchange chromatogram of the DNase digest under the conditions employed to elute the further degradation products (nucleosides, nucleotides, and nucleoside diphosphates) listed in Table II. For this reason, very small amounts of these substances can be detected if produced by subsequent degradation.

Experiments 1 and 2—The conditions of phosphodiesterase digestion in Experiments 1 and 2 were adequate for the quantitative degradation of a pancreatic DNase digest to mononucleotides. The fact that the substances in these splenic DNase digests were not completely degraded in this way, unless the monoesterified phosphate was first removed as in Experiment 2, proved that there were polynucleotides in this digest of a character different from those in the pancreatic DNase digest. This was further shown by the presence of the nucleosides in Experiment 1. With the indicated assumptions, the near-equality of the amount of nucleoside in Experiment 1 with the amount of monoesterified phosphate determined with the phosphatase led to the conclusion that all the polynucleotides in the splenic DNase digest had a 3′-terminal monoesterified phosphate. It was then postulated that the incomplete digestion by the phosphodiesterase in Experiment 1 was the result of the presence of this type of end group.

Nucleoside Diphosphates—To determine whether this resistance to complete degradation with phosphodiesterase was an absolute resistance or was only relatively great (in comparison with the digest run with the terminal phosphates removed), Experiment 3 was run with a much larger concentration of phosphodiesterase. In this case there appeared in the dinucleotide portion of the chromatogram four compounds, each with a spectrum closely resembling one of the mononucleotides. Of the compounds that might be found in the dinucleotide region of the chromatogram, only the nucleoside diphosphates would be limited to four in number and with unmixed nucleotide spectrum. That these substances were the nucleoside 3′,5′-diphosphates which would be expected from a complete phosphodiesterase degradation of polynucleotides with a 3′-terminal phosphate was proved by demonstration that the compounds with the spectra of thymidine, adenosine, and guanosine contained 2 moles of total phosphate per mole of mononucleotide absorption, and that all of this phosphate could be released as inorganic phosphate by means of prostatic phosphatase. The compound with the spectrum of cytidine was not isolated in sufficient amount for this determination.

Since the nucleoside diphosphates represented the 3′-monoesterified phosphate ends of the polynucleotide chains, and the nucleosides[7] rep-

[7] No simple reason can be advanced for the greater amount of thymidine recovered in Experiment 1 than in Experiment 2. Since these experiments were performed only once in this manner, this result may be an analytical error.

resented the end groups of the chains distal to the monoesterified phosphate, these compounds indicated that the splenic DNase had no specificity with regard to the purine and pyrimidine bases adjacent to the phosphodiester linkages hydrolyzed.

With even the high diesterase concentration used in Experiment 3, the digest was not completely broken down to nucleosides, nucleotides, and nucleoside diphosphates, as was shown by the recovery of some polynucleotides of mixed spectra. This result accounted for the low recovery of nucleoside diphosphates in comparison with the nucleosides.

Experiments 4 and 5—When the initial DNase digestion was carried to the extent shown in Experiments 4 and 5, a considerable amount of mononucleotides and small polynucleotides was formed by the action of the splenic DNase alone. The mononucleotides formed by the DNase were nucleoside 3′-phosphates. This was indicated by their elution pattern on Dowex 1-X8 resin, which was different from that of nucleoside 5′-phosphates, and also by experiments demonstrating their resistance to the 5′-nucleotidase of rattlesnake venom. Experiment 5 showed that this type of digest is extremely resistant to venom phosphodiesterase. The nucleoside diphosphate peaks could be discerned, but they were greatly obscured by undegraded polynucleotides. The nucleoside fraction was smaller than would be expected from the ratio of monoesterified phosphate to total phosphate.

Action of Venom Phosphodiesterase—A reasonable explanation for this great resistance to phosphodiesterase in Experiments 4 and 5 would be the assumption that smaller polynucleotides with only a terminal 3′-monesterified phosphate are completely resistant to the enzyme. The lesser resistance of larger 3′-terminal polynucleotides to complete degradation by this enzyme can be explained by assuming that, if the polymer chain is sufficiently long, it can be broken at some link by the enzyme to produce a polynucleotide with no monoesterified phosphate and a polynucleotide with a 3′ end group on one end of the chain and a 5′ end group on the other. The polynucleotide with no end group phosphate would be readily degraded to a nucleoside and to mononucleotides. The polynucleotide with both a 3′- and 5′-monoesterified phosphate would be further attacked from the end of the chain with the 5′-phosphate (27). As this attack continued, a 5′-phosphate end group would always be liberated to facilitate further attack by the enzyme, until finally a dinucleotide with both a 3′- and 5′-monoesterified phosphate would be produced, and this compound, unlike the simple dinucleotide with only a 3′-phosphate end group, would still be susceptible to attack and be cleaved to a mononucleotide and a nucleoside diphosphate.

SUMMARY

1. A procedure is described for the determination of the amount of monoesterified phosphate in large polydeoxyribonucleotides. This method utilizes the hydrolysis of the monoesterified phosphate end groups with prostatic phosphatase, followed by a special method of determination of the inorganic phosphate released.

2. By use of this procedure, it was found that splenic deoxyribonuclease (DNase) hydrolyzes deoxyribonucleic acid rapidly to polynucleotides with a monoesterified phosphate to total phosphate ratio of about 0.1. The hydrolysis then continues at an ever decreasing rate. No definite end point has been observed. The degradation cannot be related to any phosphodiesterases in the preparation. The characteristics of the cause of degradation remain constant with purification and with partial inactivation of the principal enzyme.

3. Secondary degradations of splenic DNase digests with prostatic phosphatase, with phosphatase plus venom phosphodiesterase, and with venom phosphodiesterase alone, and analyses of the products by ion exchange chromatography indicate that the polynucleotides formed by the splenic DNase have the monoesterified phosphate in the 3′ position. The DNase shows no preference for specific purine or pyrimidine bases adjacent to the phosphodiester linkages hydrolyzed.

4. Venom phosphodiesterase is capable of completely degrading a sufficiently large polydeoxyribonucleotide with 3′-terminal phosphate to nucleotides, a nucleoside, and nucleoside diphosphate. Small polydeoxyribonucleotides with 3′-terminal phosphates are resistant to the enzyme.

5. The splenic phosphodiesterase of Hilmoe will degrade a splenic DNase digest to deoxyribonucleoside 3′-phosphates in high yield.

Addendum; Degradation with Splenic Phosphodiesterase of Polynucleotides Produced by Splenic DNase—The splenic phosphodiesterases of Heppel and Hilmoe (28) and of Maver (samples of which were generously furnished by Dr. R. J. Hilmoe and Dr. L. A. Heppel) both degrade polyribonucleotides to 3′-mononucleotides, but the two enzymes are distinguished by differing specificities on certain substrates. Since splenic DNase yields polydeoxyribonucleotides with terminal 3′-monoesterified phosphates, such a digest might be a substrate for further degradation with these enzymes. Preliminary experiments have indicated that a splenic DNase digest, containing polynucleotides of a monoesterified phosphate to total phosphate ratio of 0.1, is highly resistant to Maver's enzyme, but is readily degraded with Heppel's enzyme to a digest containing less than 1 per cent of nucleosides and 80 per cent or more of mononucleotides which are deoxyribonucleoside 3′-phosphates (resistant to the 5′-nucleotidase of rattlesnake venom). This result supports the contention that the polynucleotides formed by splenic DNase have terminal 3′-monoesterified phosphates. This enzymatic degradation should provide a useful new route to the preparation of deoxyribonucleoside 3′-phosphates.

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ARTICLE:

A DEOXYRIBONUCLEASE FROM CALF SPLEEN: II. MODE OF ACTION

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